

# Denaturing Gradient Gel Electrophoresis (DGGE) in Microbial Ecology – Insights from Freshwaters

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## 1. Introduction

### 1.1 The importance of microorganisms in decomposition of plant-litter in freshwaters

Although freshwater ecosystems support a great diversity of life, knowledge of its total diversity is far to be complete, particularly among microbes (Dudgeon et al., 2006). Aquatic microorganisms are pivotal in several biogeochemical processes, playing a key role in the recycling of organic material, or contributing to energy transfer in food webs, since they constitute food sources to micro-flagellates, ciliates and invertebrates, which are then food items for small fishes (Allan & Castillo, 2007).

In small-forested streams, allochthonous input of coarse particulate organic matter (CPOM), from surrounding vegetation, is the major source of nutrients and energy for detritus food webs (Bärlocher, 2005; Suberkropp, 1998). Coarse particulate organic matter is mainly constituted by leaves that enter the streams and can be used by consumers and decomposers or stored or transported downstream. Leaves shed from riparian vegetation are rapidly colonized by fungi, specially aquatic hyphomycetes and bacteria (Bärlocher, 2005; Gessner et al., 2007; Suberkropp, 1998), a process known as microbial conditioning. During conditioning, microorganisms enhance leaf palatability by transforming the plant material into a more suitable and nutritious food source for invertebrate detritivores (Bärlocher, 2005; Suberkropp, 1998).

Aquatic hyphomycetes are commonly found on a wide range of plant substrates, such as leaves and wood, in running waters worldwide (Bärlocher, 2005; Gessner et al., 2007). The success of these fungi as substrate colonizers is mainly attributed to physiological adaptations to fast flowing waters (Bärlocher, 2005; Suberkropp, 1998). These include: 1) the high production rates of tetra- or sigmoid-shaped conidia, which allow an efficient attachment to substrata, also enhanced by the production of mucilage at the ends of the conidial arms (Read et al., 1992); and 2) the ability of producing a variety of extracellular enzymes, with cellulolytic and pectinolytic activity, which are able to break the major plant polysaccharides (Chamier, 1985; Suberkropp & Klug, 1980). Thus, aquatic hyphomycetes directly contribute to biotic fragmentation of plant litter. Moreover, aquatic hyphomycetes

can grow and reproduce at relatively low temperatures commonly found in temperate climates during autumn fall (Suberkropp, 1984). Bacteria are also able to produce enzymes that degrade the polysaccharides of plant litter (Burns, 1982), but its contribution to plant litter decomposition in streams appears to be lower than that of fungi, as assessed from microbial biomass and productivity (e.g. Baldy et al., 2002; Duarte et al., 2009a; Hieber & Gessner, 2002; Pascoal & Cássio, 2004). The lower contribution of bacteria to leaf decomposition can be related to the lack of invasive ability, which confines bacteria to leaf surfaces.

Fungi and bacteria are reported to have both synergistic (Romaní et al., 2006; Wohl & McArthur, 2001) and antagonistic (Gulis & Suberkropp, 2003; Mille-Lindblom & Tranvik, 2003; Romaní et al., 2006; Wohl & McArthur, 2001) interactions during leaf decomposition. Bacteria can utilize fine particulate-organic matter (FPOM) and dissolved-organic matter (DOM) released from the degradation of plant litter due to fungal and invertebrate activities (Sinsabaugh & Findlay, 1995) and from the lysis of dead fungal mycelia (Gulis & Suberkropp, 2003). In addition, bacteria are reported to grow better together with fungi than alone and to have low enzymatic activities in the absence of fungi (Romaní et al., 2006). However, both groups of microorganisms may also compete for resources. Aquatic fungi are able to produce antibiotics that inhibit the growth of bacteria (Gulis & Stephanovich, 1999) and a suppression of fungal growth was reported in the presence of bacteria (Romaní et al. 2006; Wohl & McArthur, 2001), probably due to the production of fungicides or chitinolytic enzymes.

## **1.2 Assessing microbial diversity on plant-litter in freshwaters – Traditional versus molecular approaches**

Much of the current knowledge on diversity of aquatic hyphomycetes on plant-litter has been acquired by the identification of their characteristic conidial shapes (Bärlocher, 2005; Gessner et al., 2003). Typically, leaves colonized in streams are aerated in microcosms containing filtered stream water, for approximately two days, and the released conidia are trapped on a filter, stained and identified under a light microscope (Bärlocher, 2005; Gessner et al., 2003). However, assessing the diversity of fungal species based on its reproductive ability can miss fungal taxa that are not sporulating (Nikolcheva et al., 2003, 2005). Moreover, because sporulation is often more sensitive than biomass to environmental factors, the true diversity on leaves may be underestimated when taxon identification only relies on the analysis of reproductive structures (Niyogi et al., 2002).

Studies on diversity of leaf-associated bacteria are scarce and most limited to the analysis of cultivable genera or the number of different morphotypes, after staining bacterial cells with a fluorescent dye (Baldy et al., 2002; Hieber & Gessner, 2002; Suberkropp & Klug, 1976). Suberkropp and Klug (1976) isolated bacteria on decomposing leaves, belonging to the genera *Flexibacter*, *Achromobacter*, *Flavobacteria*, *Pseudomonas* and *Cytophaga*, but few of these were able to degrade structural polymers, such as cellulose. However, the inability to generate pure cultures, for the majority of bacteria, limits the knowledge on bacterial diversity and its role in ecological processes. Therefore, traditional microbiological techniques and conventional microscopy can be insufficient to examine the composition of microbial communities and the activity of individual species on decomposing plant-litter.

On the other hand, molecular methods do not rely on the presence of reproductive stages to identify taxa and are culture independent (Bärlocher, 2007). In particular, community fingerprinting techniques, such as terminal restriction fragment length polymorphism (T-RFLP) (Kim & Marsh, 2004; Liu et al., 1997) and denaturing gradient gel electrophoresis (DGGE) (Kolwalchuk & Smit, 2004; Muyzer et al., 1993, 2004), applied to 18S rRNA gene or internal transcribed spacer (ITS) regions in fungi and to 16S rRNA gene in bacteria, respectively, have been widely used to assess fungal and bacterial diversity in environmental samples. In both techniques, DNA is extracted from mixed populations and primers are used to amplify the sequences of a specific group of organisms, via polymerase chain reaction (PCR). In T-RFLP, DNA amplification is done with one or both primers fluorescently labelled at the 5' end, the PCR products are digested with a restriction enzyme and the labelled terminal fragments are then separated by sizes and detected in a DNA sequencer (Liu et al., 1997). The number of different terminal fragment sizes gives an estimate of strains present in the community (Kim & Marsh, 2004; Liu et al., 1997). Both T-RFLP and DGGE were successfully applied to assess fungal and bacterial diversity on decomposing plant-litter in streams (e.g. Das et al., 2007; Duarte et al., 2010; Nikolcheva et al., 2003, 2005; Nikolcheva & Bärlocher, 2005) and in lakes (Mille-Lindblom et al., 2006). Details on DGGE and its application to assess the diversity of microbial decomposers of plant-litter are given in the next section of this chapter.

Quantitative real time PCR (Q-RT-PCR), which allows the estimation of copy numbers of specific genes in environmental samples (Smith, 2005), was recently used to quantify fungal and bacterial biomasses on decomposing leaves, using specific primers for the regions ITS and 16S rDNA, respectively (Manerkar et al., 2008). A great potential of Q-RT-PCR over other molecular techniques is the use of specific probes at the level of phyla, genus or even species, making the analysis of the relative contributions of each taxonomic group or species to leaf-litter decomposition possible (Fernandes et al., 2011; Manerkar et al., 2008; Suzuki et al., 2000). However, in the case of fungi, the uncertainty of the number of copies of rRNA operons per fungal cell, for the majority of species, can complicate further quantification (Manerkar et al., 2008). But Q-RT-PCR was successfully applied to determine the contribution of each fungal species, within an assemblage of 3 species, to the total biomass production (Fernandes et al., 2011). The construction of clone libraries was also useful for assessing fungal diversity in the hyporheic zone (Bärlocher et al., 2007) and on decomposing leaves in streams (Seena et al., 2008). However, such approaches are expensive, time consuming and also suffer from biases introduced during nucleic acids extraction, amplification and cloning steps (von Wintzingerode et al., 1997).

## **2. DGGE as a tool to assess the diversity of microorganisms on plant-litter in freshwaters**

### **2.1 Principles, advantages and disadvantages of DGGE**

Briefly, analysing the diversity of microorganisms on decomposing plant-litter in freshwaters using DGGE includes: 1) total DNA extraction from the plant litter that contains the mixed microbial populations using a kit for environmental samples (e.g. Ultraclean soil DNA kit, from MoBio laboratories or FastDNA Spin kit for soil, from Qbiogene; Nikolcheva et al. 2003; Duarte et al., 2010); 2) amplification of fungal or bacterial DNA using specific primers targeting the gene of interest and present in all members of the community and 3) separation of the PCR amplicons by DGGE (Fig. 1).

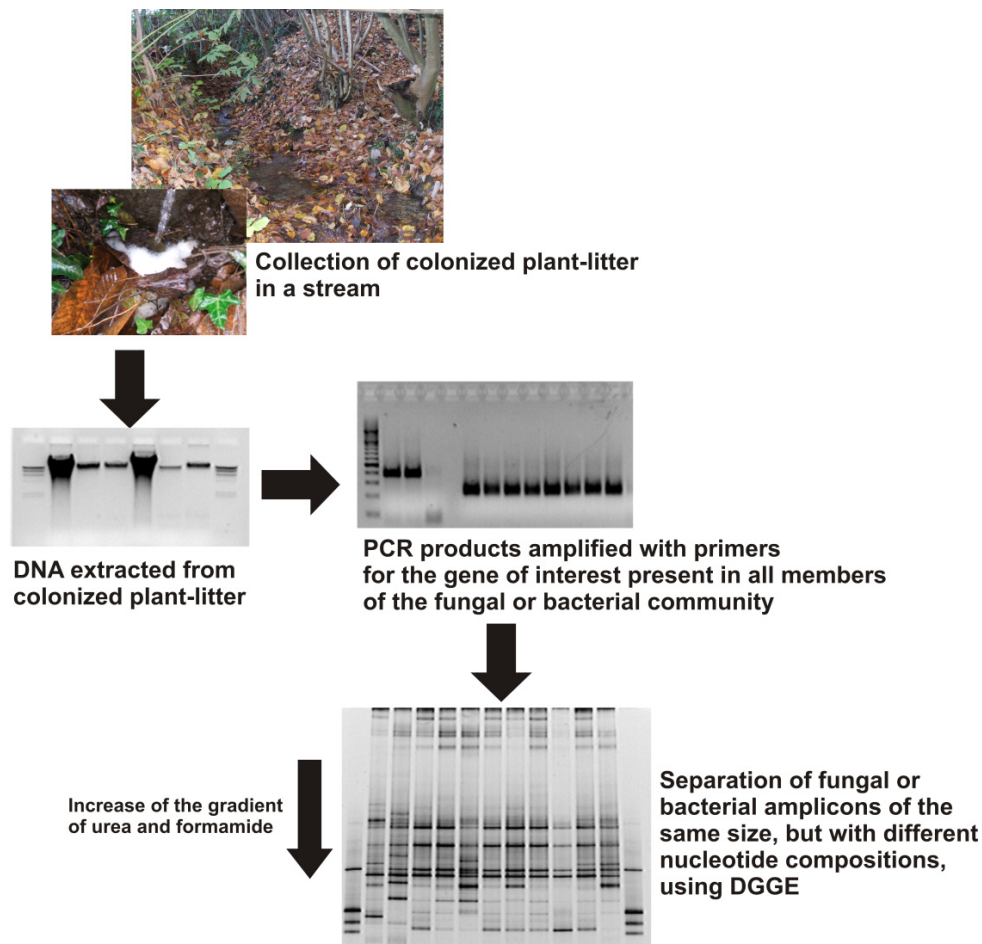


Fig. 1. Steps for DGGE analysis of the microbial diversity associated with decomposing plant-litter in freshwaters.

Amplicons of the same length but with different nucleotide compositions are separated in a denaturing gradient gel of polyacrylamide, based on their differential denaturation profile (Fischer & Lerman, 1983; Muyzer et al., 1993). The denaturing conditions are provided by urea and formamide (100% of denaturant solution consists of 7M urea and 40% formamide). Low and high denaturing solutions are prepared, mixed with an acrylamide solution and poured in a gel casting using a gradient former to generate a linear denaturing gradient (Muyzer et al., 2004). During denaturation, the two strands of a DNA molecule separate or melt at a specific denaturant concentration, and the DNA sequence stops its migration in the gel. The optimal resolution of DGGE is obtained when molecules do not completely denature, because if total denaturation occurs the PCR products will continue to run through the gel as single stranded DNA. To prevent this, a GC clamp (a stretch of DNA of 40-60 nucleotides composed by guanine and cytosine) is attached to the 5' end of one of the

PCR primers, resulting in a product with one end having a very high melting domain (Muyzer et al., 1993). The fragment containing the GC clamp when running through the gel will form a Y-shaped piece of DNA that will stick firmly on the gel when attaining its denaturing point. At the end, fragments with different melting points will migrate to different positions. After gel staining, the number of bands on the gel will be indicative of the genetic diversity of the original sample (Muyzer et al., 1993, 2004).

By using DGGE, in a span of few hours, a picture of the diversity and structure of microbial communities present in several environmental samples can be assessed, and in a lesser expensive way than other fingerprinting techniques (e.g. T-RFLP involves analysis of all terminal restriction fragment lengths obtained in a DNA sequencer). In fact, DGGE has been used for a variety of purposes such as: 1) analysis of complex communities; 2) monitoring of population shifts; 3) detection of sequence heterogeneities; 4) comparison of DNA extraction methods; 5) screening clone libraries and 6) determination of PCR and cloning biases (reviewed in Muyzer & Smalla, 1998 and Muyzer et al., 2004). In addition, a great advantage of DGGE over other fingerprinting techniques is that it is possible to obtain taxonomic information because bands can be excised, re-amplified and sequenced, and specific bands can also be hybridized with specific oligonucleotides probes (Heuer et al., 1999; Riemann & Widing, 2001). Therefore, DGGE combines the advantages of cloning, sequencing and T-RFLP (Nikolcheva & Bärlocher, 2005).

The main disadvantages of using DGGE are the same of all DNA-based techniques and include: 1) variable DNA extraction efficiencies (Theron & Cloete, 2000); 2) PCR biases (amplification errors, formation of chimeric and heteroduplex molecules and preferential amplification) (von Wintzingerode et al., 1997), and 3) introduction of contaminants during DNA isolation and PCR (Muyzer et al., 2004). In addition, the fact of only small fragments (up to 500 bp) can be separated in DGGE may limit sequence information, and minor populations can be below the detection limit (>1% of target). Different DNA sequences may have similar motilities due to identical GC contents (Muyzer et al., 2004), and, therefore, one band may not necessarily represent one species (Gelsomino et al., 1999). Moreover, possible intra-specific or intra-isolate heterogeneity of rRNA genes can give rise to multiple banding patterns for one species (Michaelsen et al., 2006; Nakatsu et al., 2000).

Having all these considerations in mind one could say that all the populations present in a habitat are not displayed in the DGGE fingerprint and, thus, the image of communities provided by DGGE fingerprinting patterns probably relates more to its structure and to the relative abundance of the main populations than to its total richness (Muyzer & Smalla, 1998). An individual discrete band refers to a unique "sequence type" or phylotype or operational taxonomic unit (OTU), which is treated as a discrete fungal or bacterial population (group of fungal or bacterial cells present in a specific habitat and that belongs to the same species). Another very important thing is the reproducibility of the gels that depends on the upstream analytical steps such as sampling, DNA extraction and amplification (reviewed in Fromin et al., 2002). All these steps should be extensively standardized. The use of reference patterns, the loading of precise amounts of DNA and the precision of the gel staining are very important to yield reproducible gels. If care is taken during these steps, identical samples loaded on a single gel will display identical patterns and different gels can be compared with a high degree of confidence (Schäfer et al., 2001; Simpson et al., 1999; Yang et al., 2001).

## 2.2 Choice of primers for DNA analysis by DGGE

Since DGGE strongly relies on PCR amplification, the choice of adequate primers for accurate characterization of microbial communities is critical (Schmalenberger et al., 2001). Bacterial rDNA (16S rDNA) and nuclear fungal rDNA (18S rDNA, ITS and 28S rDNA) are considered suitable for studying the structure of bacterial and fungal communities, respectively. Ribosomal DNA fragments to be targeted for DGGE analysis should have highly conserved, moderately and highly variable regions. Highly conserved regions can act as alignment guides and are convenient sites for anneal of universal primers, while moderately and highly variable regions allow discrimination between groups and organisms (Head et al., 1998).

Primer pair	Target	References
<b>Fungi</b>		
NS1(F)/GC fung(R)	18S rDNA (5' portion)	Das et al., 2007; Duarte et al., 2010; Mille-Lindblom et al., 2006; Nikolcheva et al., 2003
ITS3GC(F)/ITS4(R)	ITS	Duarte et al., 2008b, 2009a,b, 2010, 2011; Fernandes et al., 2009; Medeiros et al., 2010; Moreirinha et al., 2011; Nikolcheva & Bärlocher, 2004, 2005; Nikolcheva et al., 2005; Pascoal et al., 2010; Pradhan et al., 2011; Raviraja et al., 2005; Sridhar et al., 2009
<b>Bacteria</b>		
338GC(F)/518(R)	16S rDNA (V3)	Duarte et al., 2008b, 2009b, 2010, 2011; Pradhan et al., 2011
357GC(F)/518(R)	16S rDNA (V3)	Mille-Lindblom et al., 2006
984GC(F)/1378(R)	16S rDNA (V6-to-V8)	Das et al., 2007; Duarte et al., 2009a, 2010

Table 1. Primers used to assess diversity of fungi and bacteria associated with plant litter in freshwaters. ITS, internal transcribed spacer region; V3 and V6-to-V8, variable regions on 16S rDNA; F, forward and R, reverse primers.

In DGGE, the specificity of the coverage of the analysis will strongly depend on the quality of the primers chosen (Kowalchuk & Smit, 2004). Several primer sets have been developed to analyse fungal (Kowalchuk & Smit, 2004) and bacterial communities (Muyzer et al., 2004; Schmalenberger et al., 2001). The primer pairs NS1/GCfung, targeting a portion of the 5' end of the 18S rDNA and ITS3GC/ITS4, targeting the internal transcribed spacer region 2 (ITS2), have been used to characterize fungal communities on decomposing plant-litter and the diversity of fungal conidia in streams using DGGE (Table 1). Concerning bacteria, primer pairs targeting the different variable regions (V1 to V9 regions) of the bacterial 16S rDNA have been developed (Muyzer et al., 2004; Schmalenberger et al., 2001). Specifically, for assessing bacterial communities on decomposing plant-litter in freshwaters, primers targeting the V3 region (e.g. 338GC/518) (Duarte et al., 2008b, 2009b, 2010, 2011; Pradhan et al., 2011) and the V6-to-V8 regions (e.g. 984GC/1378) have been widely used (Das et al., 2007; Duarte et al., 2009a, 2010) (Table 1). In addition, before getting final amplicons to be ran in DGGE, a first PCR amplification can be used to target diversity of fungal or bacterial

taxa belonging to specific groups (e.g. actinomycetes, Das et al., 2007; discrimination of members belonging to Ascomycota, Basidiomycota, Chytridiomycota, Oomycota and Zygomycota, Nikolcheva & Bärlocher, 2004).

When analysing microbial communities on decomposing plant-litter in a low order stream, Duarte and collaborators (2010) found a similar number of bacterial DGGE OTUs with primers targeting the regions V3 and V6-to-V8. However, a higher number of fungal DGGE OTUs was found with primers targeting the ITS2 region than with primers targeting a portion of the 5' end of the 18S rDNA. The high variability of the ribosomal ITS region may provide a high level of discrimination between fungal species. Indeed, the primer pair ITS3GC/ITS4 was able to show 9 OTUs from DNA of 10 aquatic hyphomycete species, while the pair NS1/GCfung was able to discriminate only 4 OTUs (Duarte et al., 2010). In a very recent study discrimination of ITS2 regions from different *Articulospora tetracladia* strains, an aquatic hyphomycete commonly found on decomposing plant-litter in freshwaters, was even possible, suggesting that DGGE of ITS2 region might be used as a rapid and less expensive tool (comparing for example with sequencing) for assessing intraspecific diversity of aquatic hyphomycete species (Seena et al., 2010a).

### 2.3 Assessing microbial diversity on plant-litter in freshwaters by DGGE

DGGE has found applications in microbial ecology for almost 20 years (Muyzer et al., 1993), but it was just 8 years ago that it was applied to assess diversity of microorganisms on decomposing plant-litter in freshwaters (Nikolcheva et al., 2003). By using 6 different plant substrates (red maple, linden, alder, beech and oak leaves and wooden popsicle sticks), which were immersed in a stream during 1 to 4 weeks, Nikolcheva and collaborators (2003) concluded that richness of fungal species assessed by DGGE was consistently higher than that based on conidial morphology or T-RFLP analysis. However, no subsequent sequence analysis was done but the dominant phylotype on DGGE matched with the dominant sporulating species (*Articulospora tetracladia*).

The idea that fungal communities on decomposing plant-litter were mainly constituted by members of Ascomycota and Basidiomycota (e.g. Suberkropp, 1998) was questioned when Nikolcheva and Bärlocher (2004), by using DGGE and taxon specific primers, found large numbers of phylotypes of Chytridiomycota and Oomycota. The authors concluded that the combination of DGGE with primers targeting certain fungal groups facilitates a more balanced approach for studying fungal diversity in freshwaters (Nikolcheva & Bärlocher, 2004). DGGE also revealed high fungal diversity after 2-3 days of immersion in a stream (Nikolcheva et al., 2005). This fact suggests that recently fallen leaves besides carrying terrestrial fungi may promptly attract many aquatic fungi, which are not usually detected by conventional microscopic techniques in the early stages of litter decomposition (Nikolcheva et al., 2005). DGGE was also useful to detect fungal conidia in water samples from streams, and thus, might be used to check the accuracy of taxonomy and identification based on conidial morphology (Raviraja et al., 2005).

The first attempts to assess the diversity of bacteria during decomposition of plant litter in lakes (Mille-Lindblom et al., 2006) and streams (Das et al., 2007; Duarte et al., 2010) were done using DGGE; before, only morphotypes or cultivable bacteria were taken in consideration (e.g. Hieber & Gessner, 2002; Suberkropp & Klug, 1976). In addition, by using specific primers for actinomycetes, Das and collaborators (2007) were able to detect for the first time phylotypes belonging to this group of bacteria on decomposing leaves.

Further studies used DGGE to assess shifts in the structure of fungal and bacterial communities after exposure to anthropogenic stressors (e.g. Duarte et al., 2008a, 2009a,b; Fernandes et al., 2009; Moreirinha et al., 2011; Pradhan et al., 2011). For instance, DGGE proved to be a good alternative to assess fungal diversity because it is able to show several OTUs when the number of fungal reproductive structures was very low or almost absent after exposure to stressors (Duarte et al., 2008b, 2009b; Medeiros et al., 2010; Moreirinha et al., 2011; Pradhan et al., 2011).

## **2.4 Assessing effects of environmental variables on microbial community structure by DGGE**

To assess shifts on microbial communities exposed to different environmental changes, the sampling at different time points over a long period of time is often required (Muyzer & Smalla, 1998). As previously mentioned, cloning techniques are not suited for the analysis of many samples. On the other hand, by using DGGE, many samples can be processed and compared at the same time, facilitating time series analysis, assessment of exposure effects or of sites with different environmental conditions.

By using DGGE it was observed that environmental factors, seasonal patterns and time of plant-litter immersion in streams appeared to be more important than plant-litter quality for structuring microbial communities on decomposing litter (Das et al., 2007; Nikolcheva & Bärlocher, 2005). On the other hand, in lakes, although water chemistry and plant-litter properties influenced microbial community, DGGE analysis revealed that plant-litter species and nitrogen content were the factors that most affected the number of taxa (Mille-Lindblom et al., 2006). In addition, major differences were found between microbial communities on alder or eucalyptus leaves colonized in the same stream and used to feed a freshwater shrimp (Duarte et al., 2011). Moreover, microbial communities on fecal pellets produced by the shrimps were discriminated by DGGE (Duarte et al., 2011).

DGGE was sensitive enough to discriminate between reference and impacted locations by using bacterial and fungal communities on decomposing plant-litter (Duarte et al., 2009a, Sridhar et al., 2009). Indeed, multivariate analysis based on fungal and bacterial fingerprints proved to be an useful tool to detect shifts in the structure of aquatic microbial communities exposed to anthropogenic stressors in microcosms, such as metals ions alone or in mixtures (e.g. Duarte et al., 2008b, 2009b; Medeiros et al., 2011), metal nanoparticles (Pradhan et al., 2011), mixtures of metals and nutrients (Fernandes et al., 2009), and mixtures of metals and polycyclic aromatic hydrocarbons (PAHs) (Moreirinha et al., 2011). Temperature gradient gel electrophoresis (TGGE), whose principle is very similar to that of DGGE, but instead of a chemical gradient is used a temperature gradient in the gel, was also successfully applied to monitor fungal communities structure in harsh environments such as groundwater wells and heavily polluted surface waters (Solé et al., 2008).

## **2.5 Using DGGE to determine individual species densities within communities – Is that possible?**

An advantage of DGGE over other fingerprinting techniques is that the intensity of each band might provide an estimate of the abundance of specific taxa (Nikolcheva et al., 2003; Nübel et al., 1999). Band intensity might be directly related to the density of the



corresponding phylotype in the template mixture, if no bias occurred during the whole extraction-amplification procedure of the microbial genomes (Murray et al., 1998; Muyzer et al., 1993). In fact, when mycelia of two aquatic fungal species, *Anguillospora longissima* and *Clavariopsis aquatica*, were mixed at known ratios, amplified and separated on DGGE, band intensity reflected these ratios (Nikolcheva et al., 2003) and the highest band intensities on DGGE gels belonged to the species that released the largest number of spores (*Anguillospora filiformis* and *Articulospora tetracladia*) (Nikolcheva et al., 2005).

By using band intensities, Nikolcheva and Bärlocher (2004) calculated what percentage of the entire fungal community was represented by members of individual fungal groups. The authors found that Ascomycota dominated ( $\geq 75\%$  of the phylotype intensity) the fungal community on all substrates and all dates; Basidiomycota contributed up to 13% of phylotype intensity on wood and beech; Chytridiomycota were fairly common on all substrates in winter (up to 21% on wood); Oomycota were only present in the summer and Zygomycota contributed less than 1% to total band intensity (Nikolcheva & Bärlocher, 2004). Pascoal and collaborators (2010) also estimated species-specific biomasses of 4 aquatic hyphomycete species on leaf litter under zinc stress by using DGGE band intensities. Therefore band intensities on DGGE might be useful indicators of biomass of individual fungal species on plant litter, at least in assemblages with very few species, giving accurate and reproducible results (Nikolcheva et al., 2003).

## 2.6 Statistical analyses of DGGE fingerprints

### 2.6.1 Analyzing the gel in a computer-assisted program

After performing a DGGE gel, the next step is to analyze the gel with a computer-assisted program. GelCompar II and Bionumerics (<http://www.applied-maths.com/>) are among the most used programs, which allow the characterization of the banding patterns (Rademaker & de Bruijn, 2004). Briefly, by using one of these two programs the gel is processed in 4 steps: 1) definition of the area of the gel to be analyzed (including lanes); 2) correction of background noise; 3) normalization to define reference lanes, which is particularly useful if samples are run in different gels, and 4) bands (peaks) search in the fingerprints. Also the program allows other operations such as spot removal, spectral analysis, alignment of distortion bars, definition of uncertain bands and optimization and tolerance statistics (<http://www.applied-maths.com/>). After processing the gel, lanes are added to a database, a key is assigned and descriptive information can be added before further analysis. Each database entry is characterized by a unique key and by user-defined information fields (e.g. stream sampling site, sampling date, plant substrate) (Rademaker & de Bruijn, 2004).

The data from fingerprints can then be analyzed in GelCompar II or Bionumerics (see next paragraph) or exported as a band-matching table and analyzed with other statistical software (see 2.6.4). In the band-matching table, each band is assigned to classes of common bands within all the profiles, and each class of bands is described by the band position in the gel and its height (the height of the peak) or its surface (the area under the Gaussian curve approximating the band) or its relative surface (<http://www.applied-maths.com/>). Several authors use the relative surface of each band in the profile ( $P_i$ ) as a proxy of the relative frequency of each taxon (e.g. Duarte et al., 2009a; Moreirinha et al., 2011; Sridhar et al., 2009) that can be estimated as follows (1):

$$P_i = n_i / N \quad (1)$$

where  $n_i$  is the surface of the peak  $i$  and  $N$ , is the sum of the surfaces of all peaks within the profile.

Both GelCompar II and Bionumerics (<http://www.applied-maths.com/>) offer some modules to compare the structure of microbial communities on natural substrates (e.g. Duarte et al., 2008b, 2009b, Fernandes et al., 2009; Pradhan et al., 2011). Both software perform cluster analysis, which place entries in a hierarchical, bifurcating structure like a dendrogram, and ordination analyses, which place entries in a two or more dimensional space. Matrices of similarity or distance can be calculated through a variety of similarity and distance coefficients and clustering methods (<http://www.applied-maths.com/>). Details of some of these multivariate analyses will be given in section 2.6.3. Other programs, such as the NIH Image software (National Institutes of Health) can also be used to analyze microbial diversity and taxon dominance (e.g. Nikolcheva et al., 2003, 2005; Raviraja et al., 2005).

## 2.6.2 Determining taxon diversity

The generated DGGE banding pattern is an “image” of the whole fungal or bacterial community, where each individual discrete band refers to an unique “sequence type” or phylotype or operational taxonomic unit (OTU) that corresponds to a discrete fungal or bacterial population. The total number of bands ( $S$ ) can be determined and used for comparing communities (e.g. Duarte et al., 2009a; Mille-Lindblom et al., 2006; Nikolcheva et al., 2003; Solé et al., 2008). Diversity comparisons can also be done taking into account the relative intensity of each band ( $P_i$ ) (Nikolcheva & Bärlocher, 2004) to determine diversity indices (Duarte et al. 2009a), assuming that primers had the same extension efficiency during PCR (see 2.1.). Shannon index ( $H'$ ) (2) and Pielou’s equitability index ( $J'$ ) (3) can be easily calculated to describe possible changes in the dominance among DGGE OTUs using the following equations:

$$H' = -\sum_{i=1}^S P_i (\ln P_i) \quad (2)$$

$$J' = \frac{H'}{\ln S} \quad (3)$$

where  $P_i$  is the relative intensity of OTU  $i$  and  $S$  is the total number of OTUs in the profile (Legendre & Legendre, 1998).

## 2.6.3 Analyzing community structure

Multivariate analyses are the best choice to evaluate differences in community structure (Ramette, 2007), and allow the comparison of community profiles between streams, along time or exposure treatments in microcosm experiments (e.g. Duarte et al., 2008b, 2009a,b; 2010; Nikolcheva et al., 2005; Shridar et al., 2009). The most commonly used are the hierarchical analyses (e.g. Duarte et al., 2008b, 2009b, 2010; Fernandes et al., 2009; Medeiros et al., 2010) and ordination analyses (e.g. Duarte et al., 2009a; Moreirinha et al., 2011; Sridhar et al., 2009).

In hierarchical analyses, data input is a similarity or dissimilarity matrix, applied directly to banding patterns. The proximity is determined by similarity or dissimilarity coefficients, for each pair of samples, and data output is a cluster that can be illustrated by a dendrogram (Legendre & Legendre, 1998). There are several coefficients, some considering just presence/absence of bands (e.g. Jaccard, Dice or a distance coefficient such as Euclidean measures) and others based on the relative intensity of each band ( $P_i$ ) (e.g. Pearson correlation coefficient, Bray-curtis index) (Legendre & Legendre, 1998; Rademaker & de Bruijn, 2004). For constructing the cluster, the most commonly used is the unweighted pair group method (UPGMA) that uses arithmetic averages. By using cluster analyses, applied to DGGE fingerprints, several authors were able to discriminate fungal and bacterial communities: 1) in different decomposing plant substrates (Duarte et al., 2011; Nikolcheva et al., 2005); 2) along time of decomposition in streams (Das et al., 2007; Duarte et al., 2010; Nikolcheva et al., 2005) and 3) on feces of a freshwater shrimp feeding on different litter types (Duarte et al., 2011). In addition, alterations on community structure were easily detected for fungi and bacteria on decomposing plant-litter after exposure to metal ions, alone or in mixtures, and with other stressors (Duarte et al., 2008b, 2009b; Fernandes et al., 2009; Medeiros et al., 2009), and to metal nanoparticles (Pradhan et al., 2011).

On the other hand, in ordination analyses, data input is a matrix of the original data or a similarity matrix, and data output is an ordination diagram. Common ordination analyses used in microbial ecology include: multidimensional scaling (MDS), principal component analysis (PCA), principal coordinate analysis (PCoA), redundancy analysis (RDA), correspondence analysis (CA), canonical correspondence analysis (CCA) and canonical variate analysis (CVA) (Fromin et al., 2002; Legendre & Legendre, 1998; Ramette, 2007).

MDS is an ordination method that can reduce complex DGGE patterns to points into a 2-dimensional scale (Fromin et al., 2002). The higher the distance between points, the higher the differences in community compositions. By using a MDS analysis, Sridhar and collaborators (2009) showed that transplanted fungal communities resembled more those of the original stream than the recipient stream.

Both PCA and RDA are methods based on linear response models, while CA and CCA are derived from a unimodal (bell-shaped) response model (Van den Brink et al., 2003). PCA generates new variables called principal components (linear components of the original variables), explaining the highest dispersion of the samples (Fromin et al., 2002). The objectives of PCoA are also very similar to those of PCA in that it uses a linear (Euclidean) mapping of the distance or dissimilarities between objects into the ordination space and the algorithm attempts to explain most of the variance in the original data set (Legendre & Legendre, 1998; Ramette, 2007). By using PCoA of absence/presence of phylotypes from DGGE, Nikolcheva and Bärlocher (2005) concluded that plant-litter type did not affect fungal communities on decomposing leaves, but communities from 4 different litter types collected on the same date grouped together suggesting an overall seasonal trend.

An unimodal distribution of bacterial or fungal populations on decomposing plant-litter is probably closer to reality, with more individuals near their optimal environmental values (Ramette, 2007) and thus, CA and CCA analyses might be the most appropriate for analyzing these communities (Fromin et al., 2002). The choice between CA and CCA depends on what we want to answer. CCA is the direct form of CA, which means that by

using CCA the researcher can focus the analysis on the particular part of the variance that is explained by external explanatory variables (environmental data) (Lepš & Šmilauer, 2003; Van den Brink et al., 2003). Therefore in CCA, beyond a similarity matrix with biological data, an environmental matrix has also to be constructed. Care should be taken when constructing the environmental matrix since environmental data are usually not in the same units and have to be normalized, which is a procedure that removes the influence due to differences between scales or units (Ramette, 2007). Both CA and CCA ordination were already successfully applied to DGGE fingerprints derived from microbial communities on decomposing plant-litter in freshwaters. Moreirinha and collaborators (2011) were able to show, through a CA analysis, that the exposure of fungal communities on decomposing leaves to cadmium and phenanthrene altered the structure of the community, with stronger effects for those exposed to mixtures of both stressors. On the other hand, by using CCA ordination, Duarte and collaborators (2009a) found that nitrate or phosphate levels in the stream water were the factors that most contributed to the structure of fungal and bacterial communities on decomposing plant-litter (Duarte et al., 2009a).

#### 2.6.4 Case study: Responses of fungal communities on plant-litter to environmental factors

Figure 2 shows a DGGE gel of fungal communities on decomposing alder leaves at two sites of the Este River, which flows through the city of Braga located in Northwest Portugal. Este 1 is at the spring of the stream while Este 2 is located ca. 5 Km downstream, near the industrial park of Braga (Duarte et al. 2008a, 2009a; Pascoal et al., 2005). At first glance, fingerprints of fungal communities from leaves decomposing at the two sites appear to be different, but visual inspection is not enough to draw any particular conclusion (Fig. 2).

Data from some chemical and physical parameters measured at each stream site are presented in Table 2.

Parameter	Este 1	Este 2
N-NO <sub>3</sub> <sup>-</sup> (mg L <sup>-1</sup> )	0.8 ± 0.1	5.5 ± 0.1
N-NO <sub>2</sub> <sup>-</sup> (mg L <sup>-1</sup> )	0.002 ± 0.0002	0.02 ± 0.004
N-NH <sub>4</sub> <sup>+</sup> (mg L <sup>-1</sup> )	0.005 ± 0.003	0.7 ± 0.1
SRP (mg L <sup>-1</sup> )	0.01 ± 0.004	0.1 ± 0.03
pH	6.7 ± 0.07	6.9 ± 0.04
Oxygen (mg L <sup>-1</sup> )	11.1 ± 0.3	9.2 ± 0.5
Conductivity (μS cm <sup>-1</sup> )	43 ± 0.4	161 ± 2.6

Table 2. Chemical and physical parameters of the stream water at the two sites of the Este River, Este 1 and Este 2. Data are means ± SEM, N=6.

Generally, conductivity and concentrations of nitrates (N-NO<sub>3</sub><sup>-</sup>), nitrites (N-NO<sub>2</sub><sup>-</sup>), ammonia (N-NH<sub>4</sub><sup>+</sup>) and soluble reactive phosphorus (SRP) were higher at Este 2 than at Este 1, while the opposite was found for dissolved oxygen in the stream water. Values for pH were similar between the two sites.

With this specific example the following questions can be asked: 1) is aquatic fungal diversity different between the two sites of the Este River? 2) are aquatic fungal communities different along time of decomposition? and 3) what is the influence of abiotic environmental variables on the aquatic fungal communities? The first thing to do is to check if there are any differences in the diversity of aquatic fungi.

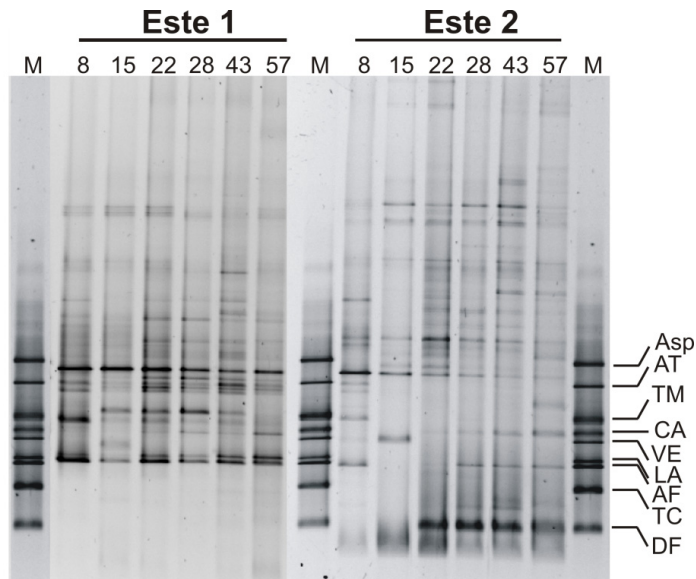


Fig. 2. DGGE fingerprints of fungal communities on leaf litter decomposing at two sites of the Este River, Este 1 and Este 2, after 8, 15, 22, 28, 43 and 57 days of leaf immersion. M, mixture of DNA from 9 aquatic hyphomycete species; Asp, *Anguillospora* sp.; AT, *Articulospora tetraccladia*; TM, *Tetraccladium marchalianum*; CA, *Clavariopsis aquatica*; VE, *Varicosporium elodeae*; LA, *Lemonniera aquatica*; AF, *Anguillospora filiformis*; TC, *Tricladium chaetocladium* and DF, *Dimorphospora foliicola*.

Table 3 shows the result of this analysis which indicate that there are not many differences in the DGGE OTUs between the two sites of the Este River, but higher numbers of bands were found in later times of decomposition for both stream sites. However, this does not tell anything about community structure on litter decomposing at both sites.

As described in the previous sub-section (2.6.3), community structure can be accessed through a range of several multivariate techniques. Let's start with a hierarchical analysis. Figure 3A shows the similarity matrix, constructed using a band-matching table from the DGGE fingerprints, exported from GelCompar II (data input), and Figure 3B shows the corresponding dendrogram (data output). In the similarity matrix (Fig. 3A) the highest the percentage between two samples, the closest the proximity between those samples. In the dendrogram (Fig. 3B) it is clear the separation in 2 groups: communities of Este 1 from those of Este 2. A closer look grouped: 1) communities of Este 2 from 15 to 57 days; 2) communities of Este 1 and Este 2, from 8 days; and 3) communities of Este 1 from 15 to 57 days. This analysis clearly shows the difference between fungal communities on leaves

decomposing at the two sites of the Este River and along decomposition time. Communities on leaves immersed for 8 days in the stream were similar at the two sites, probably because when leaves enter the streams carry terrestrial fungi that are replaced by aquatic fungi at later decomposition times.

Sample	<i>S</i>	<i>J'</i>	<i>H'</i>
<b>Este 1</b>			
8 d	15	0.86	2.32
15 d	10	0.88	2.02
22 d	17	0.95	2.69
28 d	15	0.94	2.56
43 d	18	0.93	2.70
57 d	18	0.88	2.54
<b>Este 2</b>			
8 d	10	0.89	2.06
15 d	14	0.74	1.95
22 d	22	0.77	2.37
28 d	16	0.75	2.09
43 d	21	0.79	2.41
57 d	20	0.81	2.42

Table 3. DGGE OTUs richness (*S*), Shannon diversity index (*H'*) and Pielou's evenness index (*J'*) determined from DGGE fingerprints of fungal communities on decomposing litter at two sites of the Este River, Este 1 and Este 2, after 8, 15, 22, 28, 43 and 57 days. DGGE fingerprints were transformed into a band- matching table using GelCompar II and n° of OTUs and diversity indices computed with Primer v6 software package (Primer-E Ltd., United Kingdom).

Figure 3C shows a MDS ordination diagram also constructed using DGGE fingerprints from Fig. 2. Data input corresponds to the same similarity matrix (Fig. 3A) used for constructing the dendrogram from Fig. 3B. Results are similar to those obtained using the hierarchical analysis. Nevertheless, the differences in community composition between the samples are easier to check visually using the MDS. Each point corresponds to the fingerprint obtained for each stream site in each particular sampling date along leaf decomposition. The relative distances between each point are of the same order as the relative similarities between the samples. However, none of these two multivariate techniques presented on Fig. 3 allow relating differences in community composition with differences in environmental variables.

In Fig. 4 are represented CA (Fig. 4A) and CCA (Fig. 4B) ordination diagrams of the fingerprints from fungal communities. Results from CA are very similar to those obtained using MDS; however, no similarity matrix was computed, the input matrix is the original band-matching table exported from GelCompar II.

In addition, in CA we have the option of representing in the diagram all the bands from the fingerprints and to check which are the bands most related with each particular stream site or sampling date, what can be particularly relevant if band identity is assessed through sequencing.

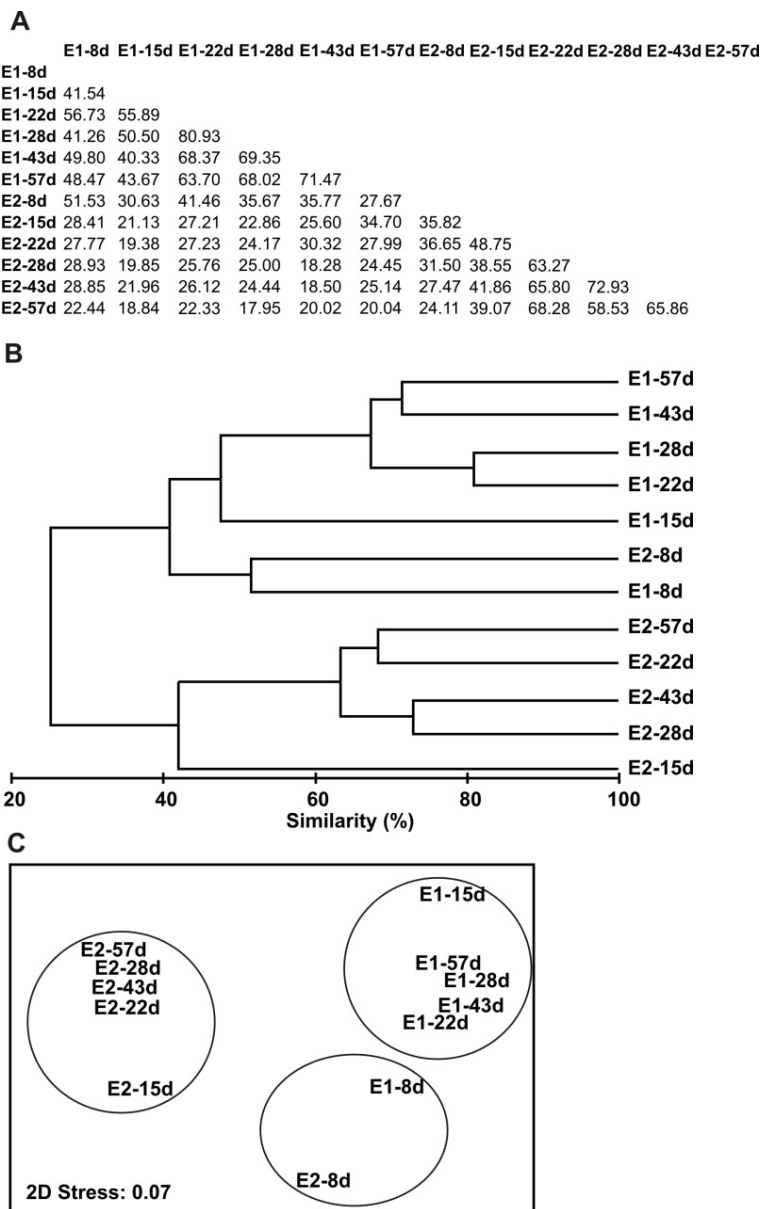


Fig. 3. Similarity matrix calculated using Bray-curtis similarity index (A), cluster analysis assessed from UPGMA (B) and MDS (C) of DGGE fingerprints from fungal communities after 8, 15, 22, 28, 43 and 57 days of litter immersion at two sites of the Este River, Este 1 and Este 2. DGGE fingerprints were transformed into a band-matching table using GelCompar II and the similarity matrix, dendrogram and MDS diagram were constructed with Primer v6 software package (Primer-E Ltd., United Kingdom).

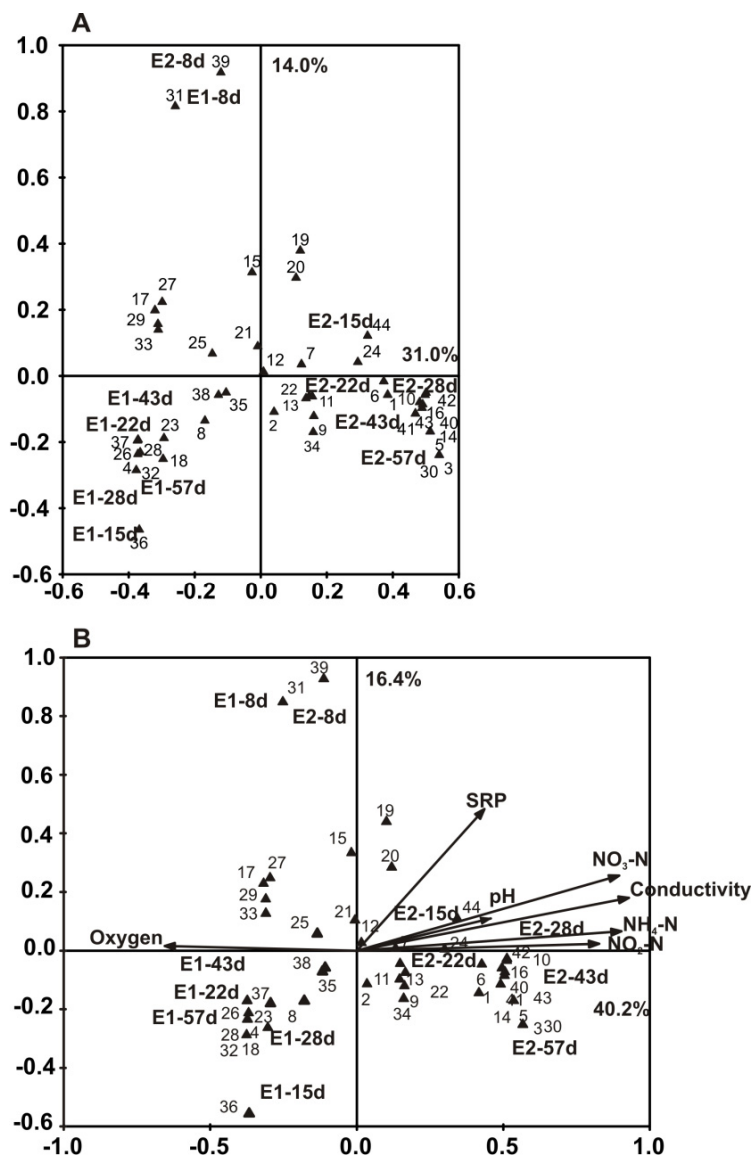


Fig. 4. CA (A) and CCA (B) diagrams for ordination of fungal communities from DGGE fingerprints after 8, 15, 22, 28, 43 and 57 days of litter immersion at two sites of the Este River, Este 1 and Este 2. Values in percentage indicate the amount of total variance explained by axes 1 and 2. In CCA, the direction of the arrows indicates the direction in which the corresponding variable increases most, and the length of the arrows reflects the magnitude of the change. DGGE fingerprints were transformed into a band-matching table using GelCompar II (Applied Maths, Belgium) and CA and CCA analyses were performed using CANOCO, version 4.5 for windows (Microcomputer Power, New York).



On the other hand, by using CCA, an additional matrix with environmental data has to be included in the analysis and will be used to explain community composition (Table 2). Since data from Table 2 are constituted by parameters displayed in different units, values were normalized. Although the CCA analysis gives similar information of previous multivariate analyses, it allowed us to determine the effects of environmental parameters on fungal community composition. Furthermore, Monte Carlo permutation tests indicated that the environmental data influenced community composition ( $P < 0.05$ ), and by observing the diagram, the environmental parameters most related to community differences are easily detected. It is clear that there is an increasing gradient of conductivity and concentrations of nitrates, nitrites, ammonia and phosphorus, from Este 1 to Este 2, while the opposite was found for dissolved oxygen. Permutation tests indicated that the differences in community composition were mainly correlated with differences in conductivity between the two sites ( $P < 0.05$ ).

### 3. Conclusions

Although DGGE was applied for the first time 8 years ago to characterize fungal communities on decomposing plant-litter in freshwaters (Nikolcheva et al., 2003), this technique has helped to circumvent much of the problems associated with the conventional techniques of microbiology and microscopy traditionally used to characterize these communities. In a span of very few years, DGGE has been valuable to: 1) assess diversity and detect shifts of fungal and bacterial populations during plant-litter decomposing in streams and lakes (e.g. Das et al. 2007; Duarte et al. 2010; Mille-Lindblom et al., 2006; Nikolcheva et al., 2003) and in very early stages of the process (Nikolcheva et al., 2005); 2) discriminate members of different fungal groups on decomposing plant-litter by using taxon specific primers (Nikolcheva & Bärlocher, 2004); 3) assess the diversity of conidia of aquatic fungi in the stream water (Raviraja et al., 2005); 4) analyse the effects of environmental factors and stressors on diversity and species composition (e.g. Duarte et al., 2008b, 2009a,b; Nikolcheva & Bärlocher, 2005; Sridhar et al., 2009); 5) assess individual contributions of fungal species or groups to total fungal biomass, using band intensities (Nikolcheva & Bärlocher, 2004; Pascoal et al., 2010); 6) test the efficiency of different primers to assess fungal and bacterial diversity (Duarte et al., 2010); 7) assess intraspecific diversity of aquatic fungal species (Seena et al., 2010a) and 8) detect shifts in aquatic microbial communities on fecal pellets of invertebrate detritivores feeding on different litter types (Duarte et al., 2011).

However, rDNA was used in all these studies, which persistence in metabolically inactive fungi does not allow us to discriminate active from inactive fungi. This was probably the main reason why the number of fungal taxa (as number of DGGE OTUs) did not appear to be much affected by the presence of pollutants (e.g. Duarte et al., 2008b; Fernandes et al., 2009; Moreirinha et al., 2011). This definitely limits the usefulness of the DGGE technique when investigating the response of communities to environmental perturbations, because rRNA genes may be detected in DNA pools for species whose growth or cellular activity is suppressed. An alternative approach for the detection of metabolically active species is to target fungal rRNA molecules extracted directly from environmental samples, based on the fact that metabolically active species will transcribe more rRNA for ribosome synthesis than inactive species (Anderson & Parkin, 2007). The direct extraction of RNA from environmental samples, followed by the synthesis of cDNA via reverse transcription

polymerase chain reaction (RT-PCR) and community profiling (e.g. DGGE, SSCP), was successfully applied to target active marine and soil bacteria (Brettar et al., 2011; Girvan et al., 2004) and fungi from different soil types (e.g. Girvan et al., 2004; Anderson & Parkin, 2007; Bastias et al., 2007). Thus, the application of DGGE using RT-PCR of portions of the rRNA will definitely provide great insights about the metabolically active and functionally important fungal and bacterial species during plant litter decomposition in freshwaters.

Moreover, the progress of the DNA barcoding project (<http://www.dnabarcoding.org/>), aiming at identifying species in a rapid and inexpensive manner by the sequence analysis of a short fragment of a single gene (Hebert et al., 2003), has stimulated microbiologists to invest in DNA sequencing. Indeed, DNA sequences from aquatic fungal species are dramatically increasing in genomic databases, particularly those from ITS region (Seena et al., 2010b). This will allow an accurate identification of species, through sequencing of excised DGGE bands, and will help to fulfil the gaps on the knowledge of fungal diversity in freshwaters.

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